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Combinations of Macrolide Resistance Determinants in Field Isolates of *Mannheimia haemolytica* and *Pasteurella multocida*▽

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Respiratory tract infections in cattle are commonly associated with the bacterial pathogens *Mannheimia haemolytica* and *Pasteurella multocida*. These infections can generally be successfully treated in the field with one of several groups of antibiotics, including macrolides. A few recent isolates of these species exhibit resistance to veterinary macrolides with phenotypes that fall into three distinct classes. The first class has type I macrolide, lincosamide, and streptogramin B antibiotic resistance and, consistent with this, the 23S rRNA nucleotide A2058 is monomethylated by the enzyme product of the *erm*(42) gene. The second class shows no lincosamide resistance and lacks *erm*(42) and concomitant 23S rRNA methylation. Sequencing of the genome of a representative strain from this class, *P. multocida* 3361, revealed macrolide efflux and phosphotransferase genes [respectively termed *msr*(E) and *mph*(E)] that are arranged in tandem and presumably expressed from the same promoter. The third class exhibits the most marked drug phenotype, with high resistance to all of the macrolides tested, and possesses all three resistance determinants. The combinations of *erm*(42), *msr*(E), and *mph*(E) are chromosomally encoded and intermingled with other exogenous genes, many of which appear to have been transferred from other members of the *Pasteurellaceae*. The presence of some of the exogenous genes explains recent reports of resistance to additional drug classes. We have expressed recombinant versions of the *erm*(42), *msr*(*E*), and *mph*(*E*) genes within an isogenic *Escherichia coli* background to assess their individually contributions to resistance. Our findings indicate what types of compounds might have driven the selection for these resistance determinants.

The *Pasteurellaceae* contain several pathogenic species the most common of which are *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*), *Pasteurella multocida*, and *Histophilus somni* (formerly *Haemophilus somnus*). These bacteria are the etiological agents of bovine pneumonic pasteurellosis, which is one of the major respiratory tract infections afflicting beef cattle (13, 31). This form of pneumonia can generally be successfully treated with macrolides or other antibiotics such as aminocylitols, amphenicols, β-lactams, fluoroquinolones, sulfonamides, or tetracyclines. However, if undetected, untreated, or inappropriately managed, the disease can develop rapidly causing high rates of morbidity and mortality in feedlots, with an estimated loss over $3 billion per year (34). Potential complications to the treatment of infections are now evident with the recent emergence of resistant *M. haemolytica* and *P. multocida* strains (16, 36). The mechanisms of resistance in these strains have been characterized for most drugs (16), with the notable exception of macrolides such as tilmicosin, tulathromycin, and gamithromycin that are used in veterinary medicine (36).

The lack of specific knowledge about macrolide resistance in the *Pasteurellaceae* contrasts with the broad general understanding of macrolide mode-of-action and resistance. Macrolides bind to the large subunit of the ribosome to block protein synthesis in bacteria; lincosamide and streptogramin B antibiotics, although structurally different, function in a similar way (23, 39). Resistance to all or to subsets of the macrolide, lincosamide, and streptogramin B (MLS_B) compounds can be conferred by efflux pumps (20, 25), drug modification enzymes (3, 38), and mutations in the ribosomal proteins (r-proteins) L4 or L22 (5, 22, 40) and by mutation (35) or methylation (26, 37) within 23S rRNA.

We report here how field isolates of *M. haemolytica* and *P. multocida* display phenotypes with distinctly different patterns of resistance to 14-, 15-, and 16-membered macrolides, indicating that resistance is being conferred by more than one mechanism. We recently showed that one resistance mechanism involves addition of a single methyl group to nucleotide A2058 in 23S rRNA by the product of the monomethyltransferase gene *erm*(42) (7). The resistance pattern in this first class of isolates is identical to the MLS_B type I phenotype, with high resistance to lincosamides and low to moderate resistance to macrolide and streptogramin B antibiotics, which is generally only found in drug-producing actinomycetes (4, 37). In the present study, we report a second class of isolates that displays macrolide resistance without concomitant lincosamide resistance, and possesses neither an *erm* gene nor methylation at nucleotide A2058. Further compounding the issue, a third class of the isolates possesses a copy of *erm*(42), although the presence of this gene alone was insufficient to explain the high resistance to macrolides.

Full-genome sequencing of a representative strain *P. multocida* 3361 from the second class of isolates reveals that it contains genes encoding a macrolide efflux pump and a macrolide phosphotransferase enzyme, and these genes are respectively named *msr*(E) and *mph*(E). These genes are arranged in

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TABLE 1. Macrolide and lincosamide MICs for the *P. multocida* and *M. haemolytica* strains used in this study[a]

Pasteurellaceae strain	MIC (mg/liter)[b]				Macrolide resistance class	Presence *erm*(42) gene	Presence *msr*(E)/*mph*(E) genes
	Tulathromycin	Gamithromycin	Tilmicosin	Clindamycin			
P. multocida 4407	0.5	0.5	4	4	Sensitive	–	–
M. haemolytica 11935	2	0.5	4	4	Sensitive	–	–
P. multocida 6052	8	8	>128	1,024	Class 1	+	–
P. multocida 6053	8	16	128	1,024	Class 1	+	–
P. multocida 6054	8	8	>128	1,024	Class 1	+	–
P. multocida 3361	64	32	32	16	Class 2	–	+
P. multocida 12602	>128	32	32	16	Class 2	–	+
M. haemolytica 12548	128	64	32	16	Class 2	–	+
P. multocida 3358	>128	64	128	>1,024	Class 3	+	+
M. haemolytica 6055	>128	128	128	>1,024	Class 3	+	+
M. haemolytica 6056	>128	128	>128	>1,024	Class 3	+	+

[a] The nomenclature for *P. multocida* and *M. haemolytica* strains has recently been changed: strains 6052 through 6056 were respectively called R11093 to R11097; strain 3358 was called R8331; strain 4407 was called R9441; and strains 3361, 11935, 12548, and 12602 have not previously been reported in the literature. All strains were tested for the presence of *erm*(42) and the *msr*(E)/*mph*(E) genes by PCR amplification and sequencing.

[b] All measurements were performed a minimum of three times and were highly reproducible.

tandem and expressed from the same promoter. The third class of highly resistant isolates is shown to contain all three *erm*(42), *msr*(E), and *mph*(E) determinants. Mapping of the locations and arrangements of *erm*(42), *msr*(E), and *mph*(E) in different strains showed that resistance is chromosomally encoded and interspersed with genes previously noted in members of the *Pasteurellaceae* and other Gram-negative bacteria. We assessed the individually contributions of *erm*(42), *msr*(E), and *mph*(E) to resistance by expressing recombinant versions of the genes within an isogenic *Escherichia coli* background. The data provide clues as to the specific types of antimicrobials that might have driven the selection for these macrolide resistance determinants.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. haemolytica* strains 6055, 6056, 11935, and 12548 and *P. multocida* strains 3358, 3361, 4407, 6052, 6053, 6054, and 12602 (Table 1) are field isolates obtained from nasal swabs of cattle in the United States; the sensitive *P. multocida* strain 4407 originated in France. All were procured from the Intervet Culture Collection (Intervet Innovation GmbH, Germany). *E. coli* strain DH1 (27), a standard *recA* laboratory strain, was used for genetic manipulations. *E. coli* strain AS19*rlmA*^{I} (17, 28) was used for MIC studies with antibiotics. The latter strain has a membrane defect that increases its susceptibility to 14-, 15-, and 16-membered macrolides and lincosamides by facilitating their intracellular accumulation; in addition, the methyltransferase gene responsible for modification of 23S rRNA nucleotide m^{1}G745 (*rlmA*^{I}) was inactivated so that this modification could not interfere with macrolide binding (23, 41). The equivalent nucleotide in *M. haemolytica* and *P. multocida* rRNAs is unmodified (7). Antibiotics used for plasmid/strain selection and MIC testing were the lincosamide clindamycin (Upjohn-Pharmacia) and the macrolides erythromycin, tilmicosin, and tylosin (Sigma), gamithromycin extracted and purified from Zactran (Merial), and tulathromycin extracted and purified from Draxxin (Pfizer). Purified gamithromycin and tulathromycin were obtained as colorless powders, and their structures were verified by liquid chromatography-mass spectrometry and nuclear magnetic resonance analyses.

rRNA isolation and MALDI-MS analysis. *M. haemolytica* and *P. multocida* strains were grown with aeration in 200 ml of brain heart infusion broth (Oxoid) to mid-log phase at an optical density of 0.6 (A_{600}). *E. coli* strains were grown in a similar manner in rich LB medium (27). Cells were harvested by centrifugation and lysed, and ribosomes were prepared by differential centrifugation (7); rRNAs were purified by phenol-chloroform extraction (32). The methylation status of nucleotide A2058 in the 23S rRNAs was determined by matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS) (2, 7).

DNA preparation for PCR and genome sequencing. *M. haemolytica* and *P. multocida* strains were grown to stationary phase in 10 ml of brain heart infusion broth prior to DNA preparation. Cells were recovered and washed before being lysed by addition of a 1/8 volume of 5% sodium dodecyl sulfate, 0.125 M EDTA, and 0.5 M Tris-Cl (pH 8.0), followed by protease and RNase A treatment. The lysate was phenol-chloroform extracted three times, and DNA was recovered from the aqueous phase by ethanol precipitation. A solution of DNA from *P. multocida* strain 3361 was nebulized to yield fragments in the size range 100 to 600 bp. The genome sequence was determined by Solexa (Illumina Genome Analyzer II) sequencing (29) with >500 times sequence coverage. The paired-end sequences were assembled using the *de novo* short read assembler Edena (14).

Screening and reconstructing the 3361 genome. BLAST searches were carried out by standard techniques (1) and software available at web databases using known and characterized macrolide resistance genes as queries. These included genes encoding the methyltransferases of the *erm* family, various *mef* and *msr* efflux genes, and macrolide modification genes, including *mph* phosphotransferases, *ereA*, *ereB*, and *ere*-like esterases, and glycosylases such as *mgt* (9, 33, 38). Alignments were made using MUSCLE multiple sequence alignment algorithms (8). After identification of the *P. multocida* 3361 contig containing the *msr*(E) and *mph*(E) genes, its sequence was extended by inverse and unpredictably primed PCR methods (21, 30), followed by Sanger dideoxy sequencing of PCR fragments. The PCR primers for this process and for the screening of strains for the *erm*(42), *msr*(E), and *mph*(E) genes are listed in Table 2.

Recombinant macrolide resistance genes in *E. coli*. The *erm*(42) gene was amplified by PCR from 3358 genomic DNA using the enzyme Phusion high-fidelity DNA polymerase (Finnzymes); likewise, *msr*(E) and *mph*(E) genes were amplified from 3361 DNA, both individually and as a tandem pair. The PCR fragment from *P. multocida* 3358 was subjected to a second round of nested PCR to introduce restriction sites for cloning into the expression vector pLJ102 (15) under the control of the *lac* promoter (7). The *msr*(E) and *mph*(E) genes were cloned individually and as a pair in a similar manner, as was the A2058 dimethyltransferase *erm*(E) that was used as a positive control to confer high MLS_B resistance (18). Recombinant plasmids were used to transform *E. coli* DH1 and were checked by restriction analysis before being moved into the AS19*rlmA*^{I} strain. In summary, five recombinant plasmids were constructed to express *erm*(42), *erm*(E), *msr*(E), and *mph*(E) individually from the *lacP* promoter and *msr*(E) and *mph*(E) as a tandem pair from a single *lacP* promoter.

Determination of MICs. The MICs of macrolide and lincosamide antibiotics were determined for the *M. haemolytica* and *P. multocida* isolates and *E. coli* AS19*rlmA*^{I} containing the plasmid *pLJ102* derivatives. Strains were grown on the media described above; IPTG (isopropyl-β-D-thiogalactopyranoside) at 1 mM was added to express the pLJ102-encoded genes. MIC measurements were carried out in microtiter plates with the addition of antibiotics in 2-fold dilution steps between 0.5 and 1,024 μg/ml. Cells were incubated at 37°C and MICs were scored after 20 h as the lowest concentration at which no growth was observed.

Nucleotide sequence accession number. The sequence data for the 10.5-kb region of the *P. multocida* 3361 genome shown in Fig. 1 has been deposited at the National Center for Biotechnology Information under GenBank accession number JF769133.

TABLE 2. Primers used in the study[a]

Primer	Sequence (5′–3′)	Application
p1	TACAAGCTTGAAGCCTGATA	Screening for *erm*(42)
p2	TCCTTATCTGCCGTTTATCT	Screening for *erm*(42)
p3	ATGCATATGAATAAAAACACT	5′-end *erm*(42) with NdeI site (underlined)
p4	GTTTATGGATCCTCTGTTAT	3′-end *erm*(42) with BamHI site (underlined)
p35	CAAGAGCTAAACAGGAGTAAA	Screening for *msr*(E) and *mph*(E)
p36	TATTTGCAACAGTGCCTCAG	Screening for *msr*(E) and *mph*(E)
p37	CAGGAGTAAATACATATGAGTT	5′-end *msr*(E) with NdeI site (underlined)
p38	CGGATAAGCTTGGCTATCAT	3′-end *msr*(E) with HindIII site (underlined)
p39	GGAAATTACATATGACAATTCAA	5′-end *mph*(E) with NdeI site (underlined)
p40	GTGCCTAAGCTTTCATATTTTT	3′-end *mph*(E) with HindIII site (underlined)
p62	TTAGTCCAACTTTTGGGGTG	Mapping contig assembly
p63	AGGATTAACAACGCGTAAGC	Mapping contig assembly
p74	GCTTGAGATAGACTAAACCC	Mapping contig assembly
p75	CGCTAAGAATCCATAGTCCA	Mapping contig assembly
p82	CAAGGACATACTGGGTTGAA	Determining chromosome integration site of exogenous sequence
p83	GGATTGACCATCATTGGTTG	Determining chromosome integration site of exogenous sequence

[a] Primers were used for PCR and Sanger sequencing to verify the exogenous DNA structure in the 3361 genome and for cloning of the *erm*(42), *msr*(E), and *mph*(E) genes to construct *E. coli* recombinants.

RESULTS AND DISCUSSION

Phenotypes of *M. haemolytica* and *P. multocida* isolates. The recent identification of *M. haemolytica* and *P. multocida* field isolates with high MICs for macrolides prompted us to investigate the resistance mechanisms in these strains. We note that at present the majority of *M. haemolytica* and *P. multocida* strains isolated from cattle with respiratory tract infections have remained susceptible to macrolide antibiotics. These are represented here by strains *M. haemolytica* 11935 and *P. multocida* 4407 that are sensitive to MLS_B antibiotics and lack all of the resistance determinants for which we tested. The antibiotic MICs that inhibited growth were tested for a range of macrolide drugs currently used in veterinary medicine and also for the lincosamide clindamycin. For all of these drugs, the MIC values for the sensitive strains fell between 0.5 and 4 μg/ml (Table 1). In contrast, the MIC values for the resistant isolates were higher by 8- to >128-fold and, on the basis of their various patterns of lincosamide and macrolide resistance, the phenotypes of the resistant isolates could be grouped into three distinct classes (Table 1).

Macrolide resistance by monomethylation of 23S rRNA. The first class of resistant isolates has an MLS_B type I phenotype and is represented here by strains 6052, 6053, and 6054. These strains displayed high lincosamide resistance (clindamycin MIC = 1,024 μg/ml) and low to moderate resistance to macrolides; notably, despite slightly elevated gamithromycin and tulathromycin MICs, they remained susceptible to these macrolides. MALDI-MS analyses of the 23S rRNAs from these strains showed that nucleotide A2058 was monomethylated, a finding consistent with the MLS_B type I phenotype. The enzyme responsible for A2058 monomethylation is encoded by the recently discovered *erm*(42) gene (*7*), and the presence of *erm*(42) was confirmed by PCR analysis of genomic DNA (Table 2).

Macrolide resistance by efflux and phosphotransferase. The second class of isolates—represented here by strains 3361,

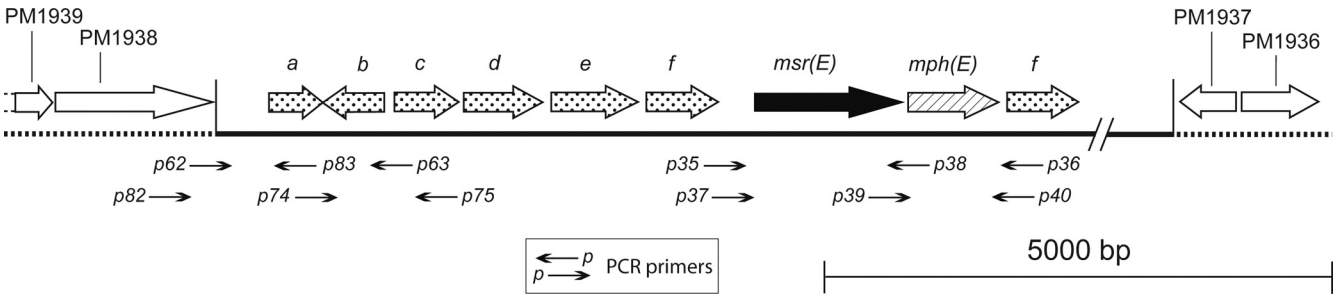

FIG. 1. Site of integration of resistance genes in the *P. multocida* 3361 genome. The *msr*(E) (black arrow) and *mph*(E) (hatched arrow) are shown as a tandem couple within a stretch of exogenous sequence inserted in the *P. multocida* chromosome (19) between the PM1937 (GenBank code NP_246876) and PM1938 (NP_246877) genes (plain arrows). Neighboring exogenous genes (dotted arrows) have sequence identity to the hypothetical proteins HSM_1733 in *Histophilus somni* 2336 (YP_001785053) and MHA_1160 (EDN74093) in *M. haemolytica* PHL213 (*a*), the tetracycline repressor protein TetR found in *Actinobacillus pleuropneumoniae* (YP_001569045) and *H. somnus* 2336 (YP_001785054) (*b*), an aminoglycoside adenyltransferase in *E. coli* (ACJ47203) and *Salmonella enterica* (ADF35688) (*c*), another aminoglycoside adenyltransferase in *S. enterica* subsp. *enterica* serovar Rissen (ABL95942) (*d*), the OXA-2 β-lactamase in *S. enterica* serovar Typhimurium (ACS54283), *Pseudomonas aeruginosa* (ABA26529), and *Klebsiella pneumoniae* (CAJ13501) (*e*), and duplicated sequences for a putative transposase TnpA in several *S. enterica* subspecies (NP_872582) (*f*). The amino acid sequence identity between 3361 and these bacteria is 100% for *a*, *b*, and *f* and 99% for *c*, *d*, and *e*. The PCR primers used for sequence verification (Table 2) are indicated by the small arrows.

12548, and 12602—possessed neither the *erm*(42) gene nor the concomitant 23S rRNA methylation and showed a different profile of macrolide resistances, while remaining susceptible to lincosamides. The resistance determinants were identified by genome sequencing of the *P. multocida* 3361 strain using Solexa Illumina GAII technology. Sequencing generated about 30 million sequence reads of 35 to 50 bp, and these were initially assembled into 64 contigs revealing that the *P. multocida* 3361 genome is composed of 2.34 Mbp and is thus ~80 kb larger than the 2.26-Mbp genome previously published for the macrolide-sensitive strain *P. multocida* Pm70 (19).

BLAST screening of the contigs using known resistance determinants as queries identified two relevant open reading frames (ORFs) that correspond to a macrolide efflux pump and a macrolide phosphotransferase. The ORFs were found on a contig of 4,075 bp (contig 44), the structure of which was confirmed by PCR and Sanger sequencing. PCR-based approaches facilitated assembly with several other smaller contigs to extend the overall contiguous sequence to 10.5 kb (Fig. 1). The efflux and phosphotransferase genes are named *msr*(E) and *mph*(E), respectively, according to the nomenclature system for macrolide resistance determinants (http://faculty.washington.edu/marilynr).

The resistance phenotypes of 3361, 12548, and 12602 in the second class of isolates (Table 1) is largely explained by the presence of the macrolide efflux gene *msr*(E) and the adjacently situated macrolide phosphotransferase gene *mph*(E). Efflux mechanisms of the *msr*(E) type are known to confer resistance to the 14-membered macrolide erythromycin and its 15-membered derivative, azithromycin (25, 38), which fits with the resistance observed for the similar compound tulathromycin. This efflux mechanism and the accompanying phosphotransferase are, however, generally regarded as being ineffective against 16-membered macrolides and lincosamides (and this conviction is confirmed here by the *E. coli* data described below); therefore, the elevated MICs observed for tilmicosin and clindamycin in the second class of *Pasteurellaceae* strains was surprising. A rigorous search of the strain 3361 genome data revealed no other genes or alterations in ribosomal components that could account for the higher MICs of tilmicosin and clindamycin. Presumably some other change, which has remained undetected by our screening approaches, has occurred in these strains. One possibility is increased expression of an intrinsic gene, such as the upregulation of an efflux pump in *Acinetobacter baumannii* that was recently reported to confer resistance to several drugs, including clindamycin (6).

Genome locations of *msr*(E) and *mph*(E). The exogenous sequence containing the resistance genes is located immediately adjacent to the 3′ end of the indigenous gene PM1938 in the chromosome of *P. multocida* strain 3361. No promoter sequence is evident between *msr*(E) and *mph*(E), and thus the genes appear to be expressed from the same promoter upstream as *msr*(E) (Fig. 1). The sequences and tandem arrangement of *msr*(E) and *mph*(E) are identical to genes that have been seen on plasmids isolated from *Enterobacteriaceae* (11–12) and *A. baumannii* (24, 43), where there were respectively called *mel* and *mph*. Several other resistance genes, including aminoglycoside adenyltransferases are distributed around *msr*(E) and *mph*(E) within the *P. multocida* 3361 exogenous sequence (Fig. 1).

The extra sequence in the genome of *P. multocida* strain 3361 has undoubtedly arisen from a collection of sources and includes genes that have previously been identified in *Actinobacillus pleuropneumoniae* and *Histophilus somni* (Fig. 1), which are related members of the *Pasteurellaceae family*. Other genes are evident that have been noted in the *Enterobacteriaceae E. coli*, *Salmonella* spp., and *Klebsiella pneumoniae*, and, further afield, in *Pseudomonas aeruginosa*. The lack of variation within these gene sequences (legend to Fig. 1) suggests that their acquisition by the *Pasteurellaceae*, and consequently that of *msr*(E), *mph*(E), and *erm*(42) (7), has been a relatively recent event. However, the locations of these genes vary within the exogenous sequences of the different *Pasteurellaceae* strains (Fig. 1) (7). It remains unclear whether these differences in location result from multiple sequence transfer events or from gene rearrangement.

Combinations of macrolide resistance genes in *P. multocida* and *M. haemolytica* strains. The resistance mechanisms in the third class of strains became evident upon PCR screening with primers designed from the *erm*(42), *msr*(E), and *mph*(E) sequences (Table 2). This third class was shown to contain all three of the macrolide resistant determinants, where *msr*(E) and *mph*(E) have retained the same tandem-pair arrangement and are located on a different contig than *erm*(42). Similar PCR analyses of the two other strain classes yielded results consistent with their phenotypes: in the first class, strains 6052, 6053, and 6054 gave a PCR product with primers p1 and p2 specific for *erm*(42), but no product with any combination of the p35 to p40 primers for detecting *msr*(E) and *mph*(E); conversely, strains 3361, 12548, and 12602 in the second class of strains gave PCR products with the *msr*(E) and *mph*(E) primers but no product with the *erm*(42) primers (Table 1).

The PCR products encompassed the entire *erm*(42), *msr*(E), and *mph*(E) genes and were sequenced by the Sanger dideoxy method. The sequences were identical to the Solexa Illumina data, and the coding region within each of the resistance genes was absolutely conserved in all of the isolates. The tandem organization of *msr*(E) and *mph*(E) was maintained in the second and third classes of strains; none of the strains contained *msr*(E) in the absence of *mph*(E) or vice versa.

The individual contributions of *erm*(42), *mph*(E), and *msr*(E) to resistance. The presence of three different macrolide resistance determinants in the *Pasteurellaceae* strains prompted the question regarding what antimicrobial compounds might have driven their selection. This was addressed by expressing recombinant versions of *erm*(42), *mph*(E), and *msr*(E) from an identical promoter in an isogenic *E. coli* background and then evaluated their contributions to resistance (Table 3).

In the absence of any resistance determinant, the *E. coli* strain used here is susceptible to erythromycin, gamithromycin, tilmicosin, tulathromycin, and tylosin (MICs of 1 to 2 μg/ml) and shows a basal MIC of 16 μg/ml for the lincosamide clindamycin. As a positive control, we constructed a recombinant version of this strain expressing the dimethyltransferase *erm*(E), which confers a type II MLS_B phenotype with high resistance to all of these compounds (Table 3). Monitoring of the rRNA methylation status by MALDI-MS showed that modification at nucleotide A2058 was close to stoichiometric (data not shown) and verified that the Erm(42) monomethyl-

TABLE 3. Expression of macrolide resistance determinants in *E. coli*[a]

E. coli strain AS19*rlmA*^{I} + plasmid	MIC (mg/liter)						Methylation status of nucleotide A2058
	Erythromycin (14-ring)	Tulathromycin (15-ring)	Gamithromycin (15-ring)	Tylosin (16-ring)	Tilmicosin (16-ring)	Clindamycin (lincosamide)	
Empty plasmid	1	2	1	2	2	16	No methylation
erm(42)	64	32	4	8	32	1,024	N^6-Monomethylated
erm(E)	>1,024	>1,024	512	>1,024	512	1,024	N^6,N^6-Dimethylated
msr(E) + *mph*(E)	128	64	8	2	4	16	No methylation
msr(E)	8	64	4	2	4	16	No methylation
mph(E)	8	2	1	2	2	16	No methylation

[a] The susceptible strain *E. coli* AS19*rlmA*^{I} harbored pLJ102 (empty plasmid), or pLJ102 containing *erm*(42), *erm*(E), *msr*(E)/*mph*(E) as a tandem pair, or *msr*(E) or *mph*(E) individually. All resistance genes were under the control of the *lac* promoter. The gene for the dimethyltransferase Erm(E) was cloned from *Saccharopolyspora erythraea* (18). MICs were measured for the lincosamide clindamycin and for erythromycin (a 14-membered ring macrolide), tulathromycin and gamithromycin (15-membered), and tylosin and tilmicosin (16-membered), which are the most commonly used macrolides in veterinary medicine. The methylation status of the 23S rRNA at nucleotide A2058 was established by MALDI-MS (7).

transferase and the Erm(E) dimethyltransferase were functioning as anticipated.

Mono- and dimethyltransferase *erm* genes confer distinctly different macrolide resistance patterns (18, 38, 42). After monomethylation by *erm*(42), the MIC of erythromycin increased the most, whereas the gamithromycin and tylosin MICs were least affected. As expected, dimethylation by *ermE* conferred high resistance to all of the macrolides. Lincosamide resistance is conferred to the same high level by both mono- and dimethylation of the 23S rRNA at nucleotide A2058 (38, 42), and the consistently high MIC values for clindamycin (Table 3) confirmed this prediction and showed that *erm*(42) and *erm*(E) were being efficiently expressed. Taken together, these findings suggest that lincosamides, and to a lesser extent erythromycin, could have exerted selective pressure for acquiring and maintaining *erm*(42). Although no lincosamides are indicated against *M. haemolytica* and *P. multocida* infections, a lincomycin-spectinomycin combination has been used in the treatment of bovine respiratory disease (10). Otherwise, lincosamide use in cattle is generally limited to the topical application of pirlimycin to treat mastitis caused by Gram-positive bacteria (10).

The *msr*(E) and *mph*(E) determinants conferred narrower resistance phenotypes than the *erm* genes. The Msr(E) efflux pump was most effective against tulathromycin (MIC of 64 μg/ml) and to a lesser extent against erythromycin and gamithromycin. In *E. coli*, the *msr*(E) efflux system was generally not effective against 16-membered macrolides and lincosamide antibiotics, although a single-step increase in the MIC for tilmicosin was observed (Table 3), indicating that the pump did excrete a modest amount of the drug. This latter observation does not explain the higher tilmicosin resistance observed in the second class of *Pasteurellaceae* isolates (Table 1).

The phenotype conferred by the *mph*(E)-encoded phosphotransferase was the least extensive of the three resistance determinants. Functioning alone, *mph*(E) conferred a modest increase in the MIC to erythromycin, but it was ineffective against the other drugs tested (Table 3). The protective value of *mph*(E) was only apparent when functioning synergistically together with *msr*(E) to confer appreciably higher resistance to erythromycin. Identical copies of the two conserved genes are encoded on plasmids in other Gram-negative species (11, 24, 43), and since their tandem organization is only advantageous in resisting erythromycin and closely related macrolides, it would seem that erythromycin was initially the agent that led to the selection and maintenance of this gene pair.

Divining the path that led to the resistance genotype found in the third class of isolates is less straightforward. It is not clear whether lincosamides or erythromycin could have exerted sufficient selective pressure to collect all three *erm*(42), *mph*(E), and *msr*(E) resistance genes, and thus the mechanisms behind their combined selection and maintenance in the same strain are still to be resolved.

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ADDENDUM IN PROOF

Since the completion of our study, another *P. multocida* strain has been reported to contain identical copies of the *erm*(42), *mef*(E), and *mph*(E) genes (K. Kadlec et al., Antimicrob. Agents Chemother. **55:**2475–2477, 2011).

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